Title: Transition from isotropic to digitated growth modulates network formation in *Physarum polycephalum*

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ABSTRACT

Some organisms, including fungi, ants or slime molds, explore their environment and forage by forming interconnected networks. The plasmodium of the slime mold *P.polycephalum* is a large unicellular amoeboid organism which grows a tubular spatial network through which nutrients, body mass and chemical signals are transported. Individual plasmodia are capable of sophisticated behaviours such as optimizing their network connectivity and dynamics using only decentralized information processing. In this study, we used a population of plasmodia which interconnect through time, in order to analyse the dynamical interactions between growth of individual plasmodia and global network formation. Our results showed how the initial conditions, such as the distance between plasmodia or their size, as well as the presence and quality of food affect the emerging network connectivity.

INTRODUCTION

Transport networks are important components of biological systems, enabling the flow of numerous vital components such as oxygen, nutrients, chemical signals, and many others. A number of species from different taxa evolved so as to grow and/or forage by forming networks: fungi (Heaton et al., 2012), slime molds (Tero et al., 2010; Alim et al., 2013; Fessel et al. 2015), ant colonies and other animals (Perna and Latty, 2014), proving the importance of transport networks in natural systems. The survival of these organisms depends crucially on the balance between two main properties of networks: the efficiency and the cost. A transport network is considered efficient, when it allows short paths and fast connections between pairs of nodes. Efficient transportation can be achieved, for instance, by having a large number of connections. However, the more connections the network contains, the more costly it is to build and maintain. Many studies have shown that the transport networks of these different organisms can reach a very good balance between cost and efficiency (Bebber et al., 2007; Buhl et al., 2009; Tero et al., 2010).

Physarum polycephalum plasmodium is a large polynucleated unicellular organism made up of a tubular network through which nutrients, body mass and chemical signals are transported (Nakagaki et al., 2000, 2001, 2004a). The shuttle streaming (the rhythmic back-and-forth flow of the protoplasm) gives the plasmodia the ability to move around few centimetres per hour (Halvorsrud and Wagner, 1998; Alim et al., 2013). In a homogeneous environment the plasmodia grow isotropically over short distances (figure 1(a)) and then they switch to a directed digitated growth (Vogel et al., 2015)(figure 1(b)). In the presence of environmental stimuli such as a light source (Hader et al., 1984), food or chemicals (Chet et al., 1977; Latty et al., 2009), the plasmodia show a directed movement toward or away from the stimulus.

Within the plasmodium body, the information used to trigger the different behaviours is not processed within a dedicated system such as the nervous system. It is distributed everywhere in the cell and comes from locally self-sustained oscillations of biochemical molecules contained in the cytoplasm (Nakagaki and Guy, 2008). This property gives the slime molds two striking features: (1) it can be cut in many pieces which can behave as autonomous plasmodia (Nakagaki and Guy, 2008), (2) several plasmodia can fuse and behave as a single plasmodium (Clark et al., 2012). Thanks to distributed computations, *P. polycephalum* plasmodia can solve complex optimization tasks in a way that is reminiscent of the so-called swarm intelligence (Bonabeau et al., 1999). Plasmodia can for example find the shortest path within a maze (Nakagaki et al., 2000), anticipate periodic events (Saigusa et al., 2008), connect efficiently several food sources (Adamatzky et al., 2011a,b; Tero et al., 2010),

make complex nutritional decisions (Dussutour et al., 2010) and learn to ignore innocuous aversive stimulus (Boisseau et al., 2016).

More interesting however, is the slime mould ability to build optimized transportation networks (Nakagaki et al., 2004a,b; Tero et al., 2010; Adamatsky and Alonso-Sanz, 2011a; Fessel et al. 2012). *Physarum polycephalum* is for example able to form a network that is comparable in efficiency, reliability, and cost to the real-world infrastructure of Tokyo's train network (Tero et al., 2010). However, in most studies conducted so far, the networks emerged from a unique plasmodium, which was offered multiple food sources. To date no study has examined networks formed by multiple plasmodia offered a unique source of food. Yet, recent results have shown that plasmodia can interact with each other in time and space (Reid et al., 2012, 2013; Vogel et al., 2015). Spatial interactions were demonstrated in a foraging context by confronting two plasmodia with a choice between two identical food sources (Vogel et al., 2015). When the two plasmodia were placed next to each other they always chose to exploit the same food source whereas when placed far away from each other food exploitation was not different from a random *i.e* they either selected the same food or different food sources. Temporal interactions were established in an exploration context by an experiment giving one plasmodium the choice between two areas, one previously explored by a plasmodium and one blank. The plasmodium showed strong avoidance behaviour toward an area explored by another plasmodium in search of food (Reid et al., 2012, 2013). Hence, one should expect that plasmodia distribution, plasmodia characteristics and presence of food in the environment might play major roles in network development and topology. In the present study, we analysed the networks formed by multiple genetically identical plasmodia under both an exploration and a foraging context focusing on the effects of inter-plasmodia distance and plasmodia size. We showed that networks formed by multiple plasmodia are optimized depending on environmental conditions.

METHODS

1) Organism and Culture

Physarum polycephalum (*Amebozoa: Myxogastria*) is an acellular slime mold that inhabits shady, cool and moist areas such as undergrowth. In the wild, it feeds on fungal spores, bacteria and other microbes, by phagocytosis. The slime molds have a haplodiplophasic life cycle composed of several states, which include fruiting bodies, spores, swarm cells, plasmodia (Keller et al., 2008). The plasmodial state, the one we used during our experiments, is a syncytium (a single cell, with many nuclei sharing the same cytoplasm).

This amorphous, typically yellow, protoplasmic mass can cover areas exceeding 5m^2 (Hausmann and Stiemerling, 1997). The plasmodium has a dormant stage, called sclerotium, to survive through unfavourable conditions such as desiccation or low temperature. Under favourable conditions it returns to a normal plasmodial state.

From 6 sclerotia of *Physarum polycephalum* obtained from Southern Victoria, Australia, we cultivated plasmodia on food medium (80mL) poured into 145-mm diameter petri dishes, incubated at 25°C in the dark. The medium was made by adding 10% weight/volume of blended oatmeal (Quaker®, Quaker Oats) to 1% agar solution.

2) Configuration of plasmodia and food

We cut circular plasmodium from the food medium cultures and transfer them in a new petri dish (diameter 145 mm) filled with 80 mL 1% agar gel. We punched holes into these agar plates, discarded the agar support and filled them with plasmodia. To study the effects of inter-plasmodia distance and plasmodia size under both exploration and foraging context, we designed 5 configurations as illustrated in figure 2. The plasmodia were disposed as the vertices of a triangular lattice, at equal distances to each of the adjacent plasmodia. In different trials we varied both the radius (r) of the plasmodia and the distance between them. We set a minimum distance between the edge of the petri dish and a plasmodium of $3/2r$ to avoid border effects during the early growth phase of plasmodia. Each of these configurations was tested in absence of food (exploration context), or in presence of a unique food source (foraging context). Under foraging context, one plasmodium was replaced with a food source (270-mm^2 in all configurations). The replaced plasmodium was either the plasmodium located at the centre or one of the peripheral plasmodia that only had three direct neighbours (i.e. on corner of the hexagonal configuration). The food sources could either be composed of oat ('low quality' food source) or egg yolk ('high quality' food source) (Reid et al. 2013). The oat food sources had the same composition as the food medium (concentration in oat: 10%). The egg food sources were prepared by adding 10% weight/volume of egg yolk and 10% weight/volume of blended oatmeal to a 1% agar solution (see Reid et al. 2013 for further details).

Overall, 25 different configurations (resulting from combinations of plasmodia inter distance, plasmodia size, presence of a food source, quality and position of the food source) were tested, with 48 replicates or more for each configuration, giving us a total of 1371 experiments.

The experiments took place in a dark room at 25 ± 2°C. Pictures were taken every 5 min using a digital camera (Canon EOS 60D with Canon zoom EF 10-22/3.5-4.5 S USM). The experiments lasted 24 h.

3) Network reconstruction

From the pictures, we extracted the dynamics of network formation, by visually tracking the appearance of connections between plasmodia on each picture of each replicate (using an homemade software © Jacques Gautrais). We describe here the rules we followed for this visual tracking. First, in our networks, the nodes corresponded to the plasmodium and the food source. The plasmodia were labelled according to their characteristics, which changed with time. Plasmodium could be labelled either as "present" or "gone". They were "present" when the plasmodium was still in its original position and "gone" when the plasmodium had completely left its original position. Food sources could be labelled as "low quality", "high quality" or "fully-exploited". "Fully-exploited" label was used when the food source was entirely covered by the plasmodia. In our network, the edges correspond to connections between plasmodia that appeared or disappeared over time. We considered that two plasmodia were connected if a direct vein of width 0.2mm or more had been established between them (Grebecki and Mockzon, 1978). We only took into account these larger veins, because the transport's efficiency of the endoplasm increases with the width of the veins (Takamatsu, 2000). Moreover, we never observed plasmodia connected only by veins thinner than 0.2mm.

4) Network analysis

We characterised the networks by computing three main types of measures: (1) measures of the connectivity at the network level at a particular time step, such as the number of connections, normalized number of connections, total network length and normalized total network length, (2) measures at the individual level that quantify the connectivity of individual plasmodium at a particular time step: plasmodium degree (number of connections to other plasmodia), normalized plasmodium degree, food degree (number of connections between the plasmodia and the food), normalized food degree and normalized shortest path to the food source, and (3) measures at the individual level that summarize the growth dynamics of individual plasmodium over time: latency to contact a neighbouring plasmodium and probability of contacting a neighbouring plasmodium through the isometric growth. Normalized parameters were obtained by dividing the experimental values of a parameter by

the same measures for a triangulated network with the same number of plasmodia as the experimental networks, but where each plasmodium is connected to all its direct spatial neighbours. This accounts for the constraints imposed by spatial embedding on network topology, which dictate that some plasmodia have six neighbours to which they can potentially connect, but other plasmodia have less.

We provide here more details about each parameter measured (Table 1). At the network level, the number of connections (table 1 Eq. 1) is the total number of direct connections established across all plasmodia in one replicate. The normalized number of connections (table 1 Eq. 2) is equal to 0 when the network contains no connection and equal to 1 when it contains all the allowed connections. The total network length (or total length of connections) (table 1 Eq. 3) is calculated as the number of connections multiplied by the inter-distance between plasmodia. The normalized total length of the network (table 1 Eq. 4) ranges from 0 to 1. Note that this last measurement and the normalized number of connections are mathematically identical (see table 1 Eq. 1-4). At the individual level, the plasmodium (or food) degree (table 1 Eq. 5) corresponds to the number of connections observed between a plasmodium (or food) and its neighbouring plasmodia. The normalized plasmodium (or food) degree (table 1 Eq. 6) ranges from 0 to 1. A network within which all plasmodia have a normalized plasmodium degree of 1 corresponds to a triangulated network. A normalized food degree of 1 means that, the food is connected to all its neighbouring plasmodia. The shortest path to the food source (table 1 Eq. 7) is the length of the shortest sequence of connections leading from one plasmodium to the food source. The normalized shortest path (table 1 Eq. 8), ranges from 0 to 1 and is computed as the theoretical shortest path in the triangulated network divided by the shortest path measured in our experiment. It is equal to 1 when the shortest path measured is equal to the theoretical one, and lower when the shortest path measured is longer than the theoretical one. The latency to contact a neighbouring plasmodium (table 1 Eq. 9) is the time it takes for one randomly chosen peripheral plasmodium to establish a connection with one of its direct neighbours. The probability of contacting a neighbouring plasmodium during the isometric growth phase (table 1 Eq. 10) corresponds to the number of randomly chosen peripheral plasmodia that contacted a neighbouring plasmodium while being in the isometric growth phase divided by the total number of plasmodium observed ($N \geq 46$ for each configuration). It would be equal to 0 if all the peripheral plasmodia observed started their digitated growth before contacting a neighbouring plasmodium.

5) Statistical analysis

The data were analysed using general linear models to compare the impact of distance between plasmodia, the plasmodia size, the presence of a food source, the quality of the food and the position of the food on the dependent variables (see supplementary information for details). All statistical analyses were conducted using R version 3.2.3. For all experiments, normality was assessed for each variable using a Kolmogorov – Smirnov test before conducting all analyses. The probabilities given in the text are always two-tailed.

RESULTS

We first described how plasmodia networks were formed in absence of food (exploration context) to investigate the effects of plasmodia size and distance between plasmodia. We next examined the effects of introducing a food source in the system.

1) Exploration context

To characterize the effect of distance between plasmodia and size of the plasmodia on connectivity over time, we plotted the absolute number of connections established between neighbouring plasmodia as a function of time (figure 3(a,e)). Since the total number of potential connections depended upon the configuration, we also plotted the proportion of connection established as a function of time (figure 3(b,f)) as well as the corresponding total length of the network (figure 3(c,g)). We first discussed the effect of distance between plasmodia, keeping the plasmodia size identical, then we considered the effect of plasmodia size keeping the distance between plasmodia constant.

a) The longer the distance between plasmodia, the lower the network connectivity

At the network level, for the shortest distance between plasmodia (1.5r), the final network was highly connected with almost all potential connections being realized, whereas less than half were established for the longest distance between plasmodia (6.0r) (figure 3(a,b)). The difference in final connectivity could be a direct effect of the censoring time of observation at 24 h. The number of connections for the longest distance (6.0r) was trending upwards at the end of the experiment (figure 3(b)), which indicates that connections might have been established after 24 hours. Thus, for the longest distance between plasmodia, the networks might still be under progress at 24 hours. In contrast, the time profiles for the others

two distances (1.5*r* and 3.0*r*) indicates that the number of connection reached a plateau, which means that the networks reached their stationary state at 24 h. Thus, the difference in connectivity between the shortest (1.5*r*) and the intermediate distances (3.0*r*) was not due to the censoring time but to a difference in the corresponding dynamics (figure 3(a,b), table S1). As a functional consequence of these dynamics, the final total length of the network was correspondingly higher for the shortest distance (1.5*r*) than for the intermediate and longest distance (3.0*r* and 6.0*r*) (figure 3(c), figure 5, table S1), and this was also true considering the normalized values of total network length (figure 3(d), table S1).

At the individual level, as expected plasmodia placed far away from each other took more time to come into contact and merge than when they were placed next to each other (latency to contact a neighbour (in minutes), mean±SE: 182.2±22.2; 424.3±18.9; 922.1±23.7 distance effect $F_{2,153}$=267.73 P< 0.001, for a distance between plasmodia of 1.5, 3 and 6*r* respectively). We observed that the plasmodia grew isotropically in all directions for a certain distance (example for the shortest distance Movie 1) then switched to a digitated growth in a limited number of directions when they approximately doubled their radius (example for intermediate distance, Movie 2, around 4 s, i.e. 7.5 h). Thus, for the shortest distance between plasmodia, all plasmodia connected their neighbouring plasmodia while they were still in the isotropic phase (table 1 Eq. 10, probability of contacting a neighbour while being in the isotropic growth phase: 1.0). In contrast, for intermediate distance (3*r*), some plasmodia switched to a digitated growth before connecting all their neighbouring plasmodia (table 1 Eq. 10, probability of contacting a neighbour while being in the isotropic growth phase: 0.82) and thus failed to connect with some of their possible neighbours. This transition from isotropic to digitated growth had the strongest consequences for the longest distance between plasmodia. None of the plasmodia contacted a neighbour while in the isotropic growth (table S2). This accounted for the lack of connectivity at 24 h beyond monitoring censoring (example for the longest distance Movie 3, also around 4 s). In terms of plasmodia connectivity, this translated into a decreasing plasmodium degree with increasing distance between plasmodia (table 2, mean normalised plasmodia degree distance, effect $F_{2,150}$=75.17 P< 0.001).

In short, for a fixed initial size of plasmodia, we showed that the distance between plasmodia had a strong impact on the rate at which plasmodia connected to each other, so that the connectivity at 24 h was inversely correlated with the distance between plasmodia (figure 3(a-d); figure 4(a)). The transition between isotropic and digitated growth that happened approximately when the plasmodia had doubled their radius, translated in a transition at the

level of global network properties. A distance between plasmodia lower than twice the radius favoured early connections with a large number of direct neighbours giving a highly connected network, whereas distance between plasmodia longer than twice the radius impeded connections between neighbours, leading to weakly connected networks.

b) The smaller the plasmodia, the higher the network connectivity

At the network level, we observed that the dynamics of network formation always converged to a stationary state whatever the initial size of the plasmodia (figure 3(e-h)). The smallest plasmodia network displayed the largest connectivity among the configurations under study (figure 3(e-f) and figure 4(b), table S3) and covered the largest normalized total length of the network (figure 3(g-h), table S3). For the intermediate and the largest plasmodia network, both dynamics of growth were similar and led to comparable normalized number of connections between neighbours over time (figure 3(f), table S3), meaning identical networks (albeit with a doubled length scale, figure 3(g)).

At the individual level, we observed that the largest and the intermediate plasmodia grew in an isotropic phase for almost 7h (examples: for the intermediate size plasmodia Movie 2, the largest plasmodia Movie 4, mean±SE 403.2±20.2 min. and 428.8±14.3 min. for the largest and the intermediate plasmodia respectively) and more than doubled their radius during this isotropic growth. In contrast, the smallest plasmodia (50mm^2) doubled their radius in about half the time *i.e.* 3 h (example for the smallest plasmodia Movie 5, 187.5±5.7 min. $F_{1,165}$=64.70 P< 0.001). The radius being 9.3mm, 5.6mm and 4mm respectively for the largest, the intermediate and the smallest plasmodia, the distance covered by the isotropic growth does not scales linearly with the size of the plasmodia. The largest and the smallest plasmodia are quicker than the intermediate plasmodia (speed: 0.021, 0.014 and 0.021mm.min-1, respectively for the largest, the intermediate and the smallest plasmodia). The smallest plasmodia connected their neighbours earlier than the intermediate and the largest plasmodia, while still being in the isotropic phase (Table 1 Eq. 10, probability of contacting a neighbour while in isometric growth: 1.0; 0.82; 0.84 for 50, 100 and 270-mm^2 respectively, table S2; table 2, latency to contact a neighbour, effect of the size $F_{2,165}$=55.44 P<0.001). In terms of plasmodia connectivity, this translates into the highest plasmodia degree for the smallest plasmodia (table 2, mean normalised plasmodia degree, size effect $F_{2,157}$=23.67 P<0.001 respectively).

Interestingly, the smallest plasmodia separated by a relative distance of 3.0*r* produced the most connected network when considering all configurations tested (figure 3(b) and figure

3(f)). This remained true even if absolute metric lengths were considered (figure 3(c) and figure 3(g)). This corresponds to a clear outlier in figure 5.

2) Foraging context

a) *The presence of a food source stimulates plasmodia relocation*

In absence of food, we almost never observed plasmodia relocation, all the plasmodia stayed in their original position. On the contrary, when we introduced food in the system irrespective of its quality (low or high) and its position (centre or periphery) plasmodia were sometimes observed leaving their original position to reach the food source instead of maintaining a long connection with the food source. This typical behaviour can be seen in Movie 6 and 7 for the longest distance between plasmodia (6*r*). In this example, the plasmodia first displayed the characteristic switch from isotropic to polarized growth, as described above (ca. 4 s, i.e. 7.5 h, Movie 6,7), then contacted the food ca. 22 h (11 s) and completely leave its original position ca. 33 h (16.5 s). Such relocations were clearly stimulated by the presence of food, but they also mostly occurred when the distance between the plasmodia and the food was the longest (proportion of plasmodia whatever their size leaving their original spot in presence of food: 0.00, 0.02 and 0.37 for 100-mm^2 and distance between the plasmodia of 1.5, 3 and 6*r* respectively; corresponding proportions in absence of food were: 0.02, 0.01 and 0.08, df = 2 χ^2 = 132.5 P < 0.001). As most of our analyses were based on the assumption that plasmodia never moved from their original position and only the connections evolved, we performed the analysis of connections dynamics in presence of food up to the time when the food had been completely covered by plasmodia to limit the number of plasmodia leaving their original position.

b) *The presence of a food source enhances connectivity*

First focusing on configurations where the food was placed at the centre, we investigated how the properties of the emerging networks were affected by the presence and the quality of the food.

At the network level, when we considered the distance parameter and focused on the connectivity of the network for the longest distance (6*r*), we noted a large increase in connectivity when we introduced a high quality food (Table 1 Eq. 2; mean connectivity: 0.71, 0.35 and 0.44 for high quality food, low quality food and no food respectively, figure 4(c), figure 6(a-c), table S4). There was no noticeable difference regarding the intermediate distance configuration (3*r*) and a slight decrease in connectivity for the shortest distance

configuration (1.5*r*) when we added a food source. When we considered plasmodia size, the connectivity for the smallest plasmodia decreased in presence of a food source from 0.96 in absence of food to 0.73 with a low quality food and 0.75 with a high quality food (figure 6(e-g), table S5) while keeping a short path to the food (figure 6(h)). This indicates that when a food source was present the network was optimized to connect all the plasmodia to the food, avoiding the "extra" connections between plasmodia (figure 4(c), figure 6(h)). All in all, the differences in connectivity observed under exploration context due to the distance between plasmodia or due to the size of the plasmodia vanished in presence of a high quality food (figure 4(c), figure 6(b,f), table S4, S5) and the connectivity was about 0.8 for all configurations. For almost all configurations, the global normalized shortest paths to the food (a proxy for the efficiency to connect the food source) were enhanced in presence of a high quality food source when compared with a low quality food sources (figure 6(d,h), table S4 and S5). In other words, plasmodia had a better access to food when the food was of high quality than of low quality. The effect was the strongest when the distance between plasmodia was the longest (figure 6(d)).

At the individual level, we focused our attention to the number of plasmodia directly connected to the food (normalized food degree; Table 1 Eq. 5 & 6). We noticed that for the longest distance between plasmodia (6*r*), the digitated growth was clearly biased by the presence of a high quality food source as pseudopods progressed directly towards the food (Movies 8, 9 to be compared with Movies 6, 7 mentioned above). Almost all the plasmodia were connected directly to the food so that the network converged rapidly to a star-like pattern in almost all replicates (table 3, figure 4(c), figure 7(a)). This was not observed with a low quality food source or in absence of food (table 3, figure 7(a), table S6). The attraction toward the food was even noticeable for the configuration with the shortest distance between plasmodia (1.5*r*) even if the connections between plasmodia established fast during the early isotropic growth phase (table 3, table S6). For the shortest distance (1.5*r*) this food effect was also true for low quality food source. This food-induced centripetal growth of the pseudopods where each plasmodium was directly connected to the food source was observed also when considering plasmodia size for the intermediate plasmodia (table 3, figure 7(b), table S6) and for the largest plasmodia (mean normalized food degree 0.85, 0.72 and 0.73 for high quality food source, low quality food source and no food respectively, figure 7(b), table S6) where the growth was enhanced towards the high quality food source at the end of the isotropic phase. This was however not observed for the smallest plasmodia, which were already highly connected in absence of food (table 3, figure 7(b), table S6).

c) *The position of the food and its quality affect network connectivity*

We finally tested the impact of the location of the food within the network, comparing the configurations with the food at the centre with configurations with the food at the periphery.

First considering only low quality food source, we found for the intermediate distance between plasmodia ($3r$), that the connectivity was higher when the food was located at the periphery than in the centre (figure 6(b), table S7, S8). Moreover, when the food was located at the periphery, it was more efficiently connected to all plasmodia than when it was located at the centre (figure 6(d), table S7, S8). This difference appeared to stem from a differential rate of connectivity of plasmodia with each other and with the food, which promoted connectivity between plasmodia before the exploitation of the food in the first case (example Movie 10). In this example, the network between plasmodia was already nearly close to full triangulation when the plasmodia adjacent to the food started to exploit it. As a consequence, almost all the plasmodia got connected to the food at once. On the contrary, when the food was located at the centre of the set-up (example Movie 11), the food was reached by the adjacent plasmodia at a stage when only a small fraction of connections among plasmodia were already established. Since plasmodia connected to the food tended to stop their explorative behaviour, this early connection prevented them from building a denser network. In short, neighbouring plasmodia connected the food rapidly, which impeded the formation of further connections. A similar significant effect, though less strong, was also observed for the longest distance between plasmodia ($6r$) (figure 6(d), table S7). For the other three configurations in which the plasmodia were closer to each other, the plasmodia had time to connect to each other before connecting the food, and no difference between central and peripheral location of the food was observed.

Second, considering only high quality food source, we found that the difference, due to the difference in food location, was no longer present for the intermediate distance ($3r$) between plasmodia (figure 6(d), table S7, S8). As a consequence of the preferential growth of plasmodia toward the high quality food source discussed earlier, the connection to the food established fast, before the network got triangulated, independently of food location. However, when we considered the longest distance ($6r$) between plasmodia, we found that the food was shared less when located at the periphery than when located at the centre (example Movie 12, figure 7(c)). The connectivity displayed a dramatic drop when the food was placed at the periphery (mean normalised number of connections: 0.71 and 0.23 for the food in central and peripheral position) (figure 6(b)). In this case, due to the effect of the transition to digitated growth combined with the directional bias towards the food, the 3 plasmodia that were the closest to the food rapidly oriented toward the food and established a connection

with it. As a side effect, they stopped their growth towards neighbouring plasmodia including the 3 plasmodia far from the food source, and the network remained disconnected in almost all cases (39 cases out of 48). For the other four configurations in which the plasmodia were closer to each others, the plasmodia had time to connect to each other before connecting the food, and no difference between central / peripheral location of the food was observed (figure 7(d)).

DISCUSSION

In our study we analysed the networks produced by *P. polycephalum* under exploration and foraging contexts. We examined networks formed by multiple plasmodia placed simultaneously in the same environment and we investigated whether this networks built from a collective of plasmodia tended to be optimal. Our aim was to understand how plasmodia interactions at an individual level determine the structure of the network produced at a global level.

In plasmodia of *P. polycephalum*, exploration follows two phases: first the plasmodium grows in all directions with an isometric rounded shape for a certain time and then they start to form pseudopods ended with fanlike structures (Baumgarten and Hauser, 2014; Latty et al., 2009; Vogel et al., 2015). Here, we showed that the transition from isotropic to anisotropic growth played a major role in the resulting networks when we varied the plasmodia distribution. First, we showed that the network connectivity increased when the metric distance between plasmodia decreased (figure 5). Plasmodia placed close to each other contacted their neighbouring plasmodia while in the isotropic phase, maximizing their chance to contact all their neighbours. On the contrary plasmodia placed far away from each other could only contact their neighbouring plasmodia while in the anisotropic growth, lowering drastically their chance to contact all their neighbours. Second, we also showed that the network connectivity depended on the size of the plasmodia. We demonstrated that small plasmodia grow faster during the isotropic phase than larger plasmodia, covering a longer distance and thus produced more highly connected networks.

Our results show that differences in the network topologies observed could be traced back to the transition from isotropic to anisotropic growth. Here we indirectly manipulated the timing of this transition by manipulating distance between plasmodia and plasmodia size. Another way of manipulating the onset of the anisotropic growth would be to use different strains. A recent study (Vogel et al. 2015), showed that the duration of the isotropic growth varies between strains. In our study, we used the Australian strain, which has been shown to

have the longest isotropic phase and the slowest growth rate. In Vogel et al (2015), the authors used two other strains, the Japanese and the American one. The Japanese strain had the same isotropic phase as the Australian strain but its growth rate was higher. The American strain, on the contrary, showed virtually no isotropic phase and grew directionally. Thus, using these different strains, in the same experimental conditions should lead to different network topology. We expect that the longer the isotropic phase the higher the connectivity. We quickly tested this hypothesis using the American and the Japanese trains in the configuration where 100-mm^2 plasmodia were 3r distant (table 1, N=4 replicate for each strain Fig 8). We showed that, as expected, the Japanese strain which had a similar timing regarding the transition from isotropic to anisotropic phase as the Australian strain, built a highly connected network while the American strain which had no isotropic phase built a less connected network (mean normalized number of connections: 0.81 and 0.20 for the Japanese and the American strain respectively).

Another major parameter that played a role in the final network topology was the environmental context in which the plasmodia grew. The introduction of food in the system had a strong effect on the local patterns of connections and in turn resulted in changes in network connectivity. These differences depended on both the quality and the position of the food. Connections between a plasmodium and a food resulted from one of the plasmodia growing, exploring the surroundings and finding the food. This means that the plasmodium had to cover all the distance from its original location to the food source. On the contrary, connections between two plasmodia arose from two simultaneously growing and searching organisms. In that later case, the plasmodia had to cover only half the distance to contact it neighbours. In our experiments, this led to a complete relocation of the plasmodium from their original locations to the food source.

The quality of the food had also a strong impact on the plasmodium growth. We noticed that plasmodia placed close to high quality food source directly moved toward the source. This was not observed, with a low quality food source. These results are in accordance with previous observation showing that plasmodia can detect a high quality food source from further away than a low quality food source (Reid et al. (2013) and prefer to move toward another plasmodia than toward a low quality food (Vogel et al 2015). In our experiments this resulted in plasmodia growing directly toward the high quality food, contacting neighbouring plasmodia on their way. Thus, the generated networks were highly connected around the food with a typical star-like pattern. Interestingly, while the connectivity increased around the food source, it decreased away from it. When a plasmodium explores is environment, it leaves behind a thin translucent slime layer that has a repulsive effect on other plasmodia (Reid et

al., 2012). If during its exploration of the environment, it finds a food source, it stops exploring its surroundings to direct all its mass towards the food, leaving only this repulsive slime around its original position. These "no go zones" might explain the reduction in the number of connections we observed away from the food source. Under exploration context, this effect was absent as long as the plasmodia were in isotropic phase allowing the emergence of a fully connected network and an efficient exploration of the environment.

Physarum plasmodia are renown for forming efficient transportation networks (Nakagaki et al., 2000; Tero et al., 2010; Alim et al., 2013). Here we showed, that patterns of growth observed at the individual level had large consequences on network connectivity. Starting from similar arrangements of plasmodia we obtained different connectivity depending on plasmodia size, plasmodia inter distance and the presence, the position and the quality of food. So, can we consider our networks as efficient?

In a configuration in which the plasmodia were far away from each other, establishing a connection between two plasmodia seemed more costly. A plasmodium needed to cover a long distance to encounter another plasmodium. Most of the time, they encountered each other by chance during the non-directional digitated growth phase. As a consequence, once the link established between them, they need to maintain a long and convoluted vein. Accordingly, an efficient network when the inter-plasmodia distance was long should have been to minimize the number of redundant connections, which is precisely what we observed in our experiment Conversely, for short inter-plasmodia distances, the cost of connections appeared lower. The plasmodia encountered each other always during the isotropic phase; hence the links established between them were as short as possible. In situations where the link cost is small, it may become optimal to establish a large number of connections with multiple neighbours to favour fast information transfer in response to environmental conditions, such as the discovery of new food source. In our experiment, when the distance was short between plasmodia we observed an almost fully connected network. Therefore, depending on distance between plasmodia different type of networks might appear optimal.

The differences between networks built in the presence and in the absence of food could respond as well to different optimization requisites. For all organisms, acquiring and saving energy is essential for their survival. All plasmodia that were introduced in our set-up were clones; hence it was in their best interest to share information and nutrient. We showed that the networks built by plasmodia in foraging context included fewer connections and were therefore less costly. Moreover, almost all plasmodia were connected to the food via the shortest path possible. Since the driving force for transportation is the variation in hydrostatic pressure along the tube, the hydrodynamic theory implies that plasmodia should concentrate

their protoplasm into few thick and short tubes which are, in principle, the most effective for nutrient transportation (Takamatsu, 2000). By forming only few thick and short tubes, the population of plasmodia could optimize food intake by maximizing the area of protoplasm available for absorbing the food and by facilitating intracellular communication between all plasmodia. Accordingly, plasmodia located far away from the food source chose to leave their original position and moved toward the food rather than try to keep long links with the food.

The transport networks built by plasmodia in presence of food were analysed when the networks were fully developed. To better understand how these network topologies emerged from isolated entities it would be interesting to study the dynamic of the network formations using a percolation paradigm. A percolation transition is observed when a large and well-connected component spontaneously emerges from multiple isolated fragments. In a previous study, Fessel and collaborators (Fessel et al., 2012) found that isolated microplasmodia fuse to form a macroplasmodia following a percolation transition driven by a unique parameter q, which correspond to the proportion of unconnected nodes divided by the proportion of degree 3 nodes. Thus, it will be interesting to see if the percolation transition in our experiments is also driven by the parameter q and if this parameter is sensitive to the configurations.

P. polycephalum plasmodia are an example of biological system in which complex behaviour emerges from decentralized interactions among simple units. In higher organisms, like insects or vertebrates, such an integrated approach is often obfuscated by the fact that the units themselves that compose the system are already extremely complex, composed of cells, tissues and complex organs such as the brain. In contrast, *P. polycephalum,* as a unicellular organism, is composed of fewer biological levels, which can be observable, and offers specific opportunities to understand distributed information processing on a molecular basis in an ecological perspective. Our work identifies a number of factors in the growth of *P. polycephalum* plasmodia that play a role in the emerging organization of plasmodial networks. Future work should aim at understanding how the 'behaviour' of individual plasmodium is implemented in terms of the physics of cytoplasmic growth, the formation of digitations and the molecular mechanisms involved in the response to physical and chemical stimuli.

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FIGURES

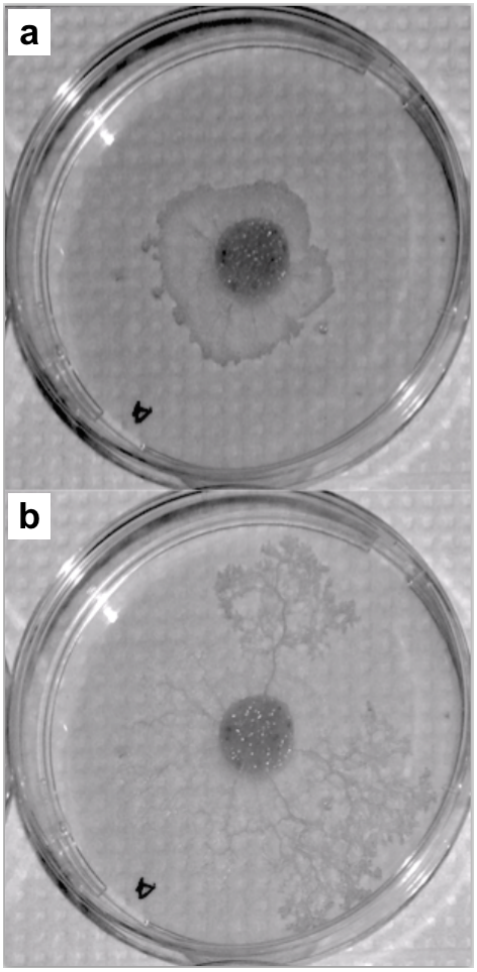

Figure 1: Pictures of the 2 growth phases of a plasmodium. (a) the isotropic growth and (b) the digitated growth.

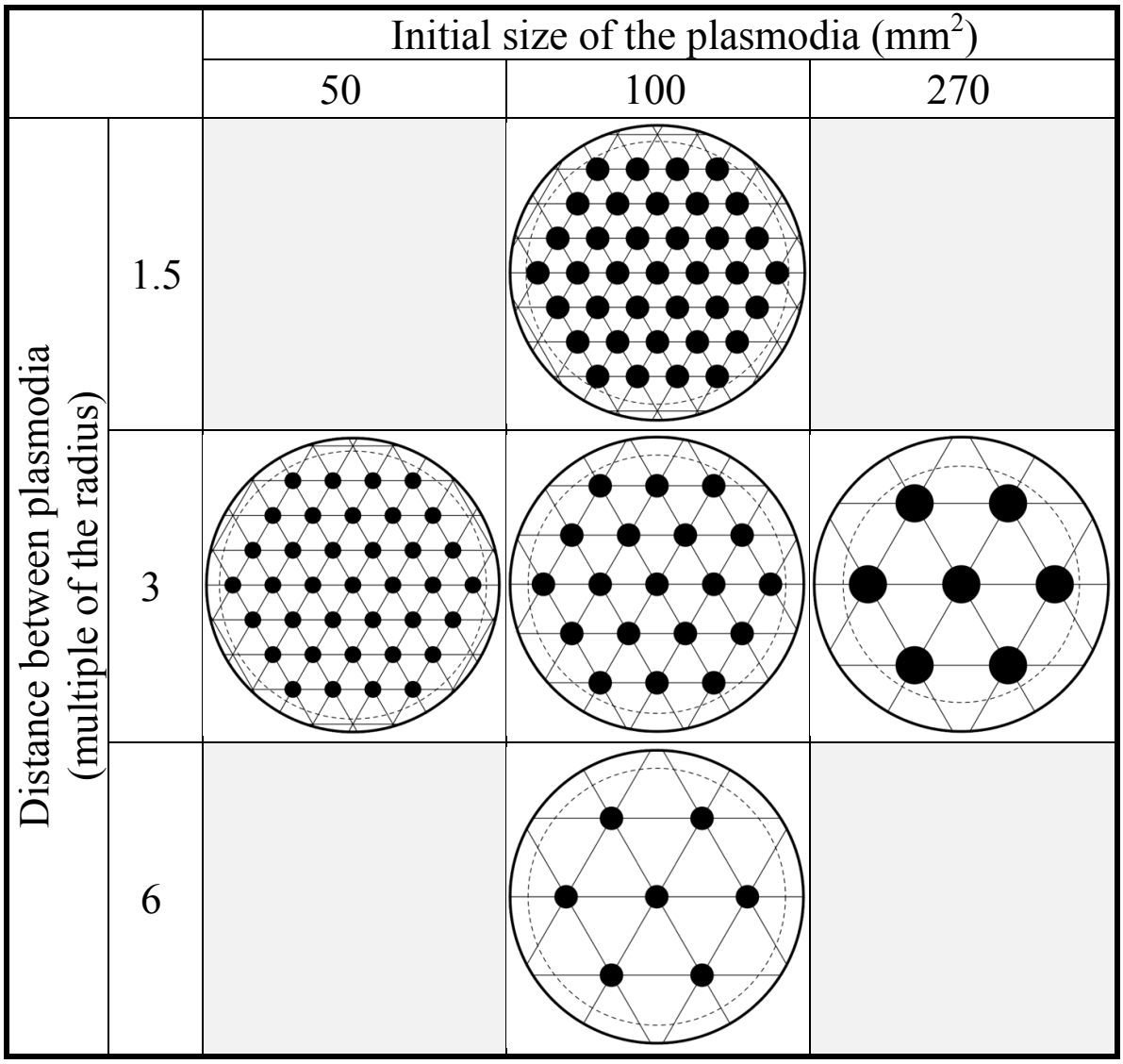

Figure 2: Geometric configurations used to test for the effect of distance between plasmodia and plasmodia size on the network produced. The black disks represent the original position and size of each plasmodium under exploration context (no food). The black lines correspond to the triangular lattice and the dash circle represents the minimal distance to the edge of the petri dish. (N=48 for 1.5*r* 100mm^2, N=52 for 3*r* 50mm^2, N=57 for 3*r* 100mm^2, N=51 for 3*r* 270mm^2 and N=48 for 6*r* 100mm^2).

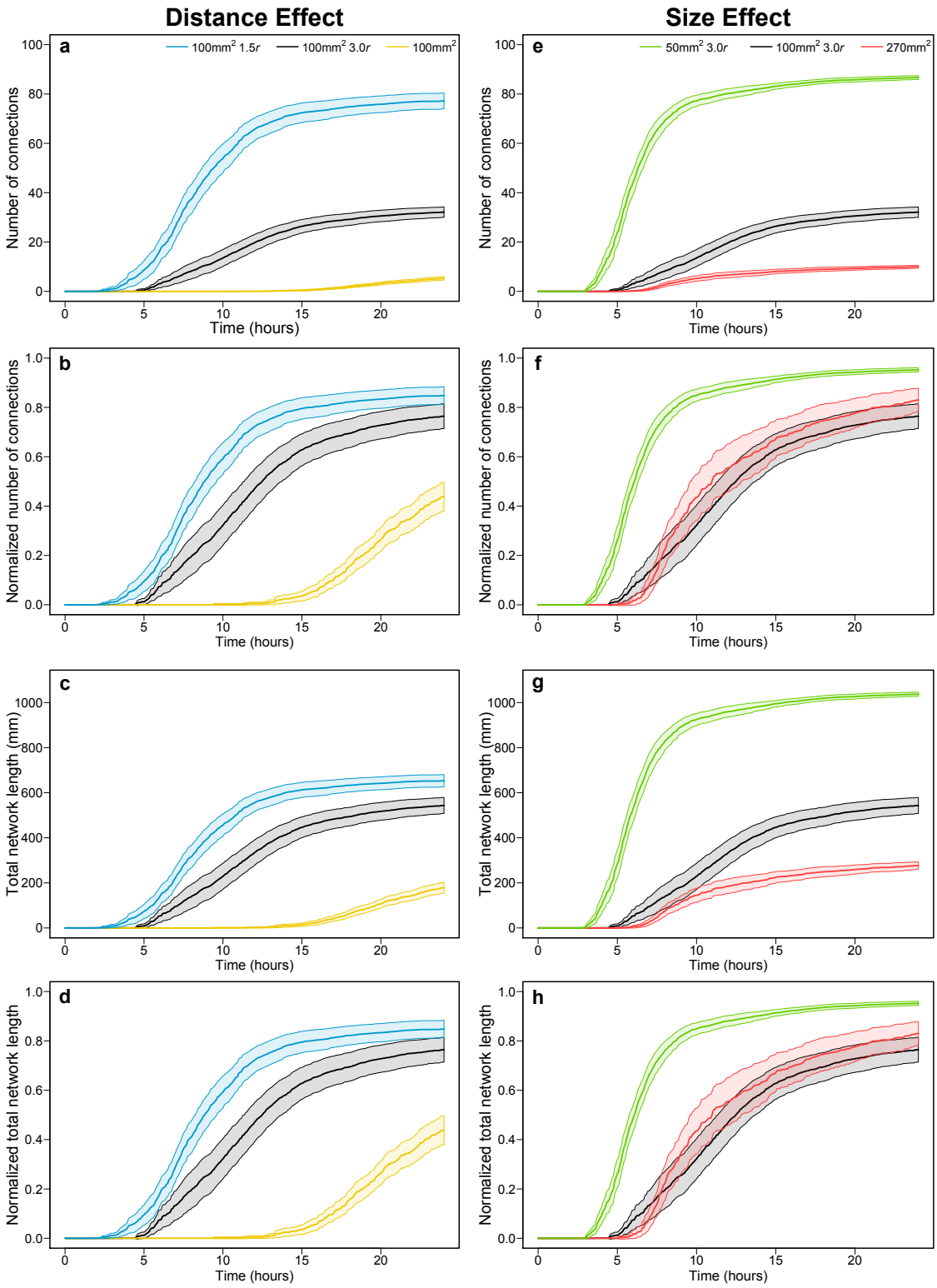

Figure 3: Dynamic of the networks formation for the 5 configurations under exploration context. Number of connections (a and d) (Table 1 Eq. 1), normalized number of connections and (c and e) (Table 1 Eq. 2) total length of the network (c and f) (Table 1 Eq. 3) and normalized length of the network (Table 1 Eq. 4) when we varied the distance between plasmodia (a-d) or the size of the plasmodia (e-h). Error bars indicate ±95% CI.

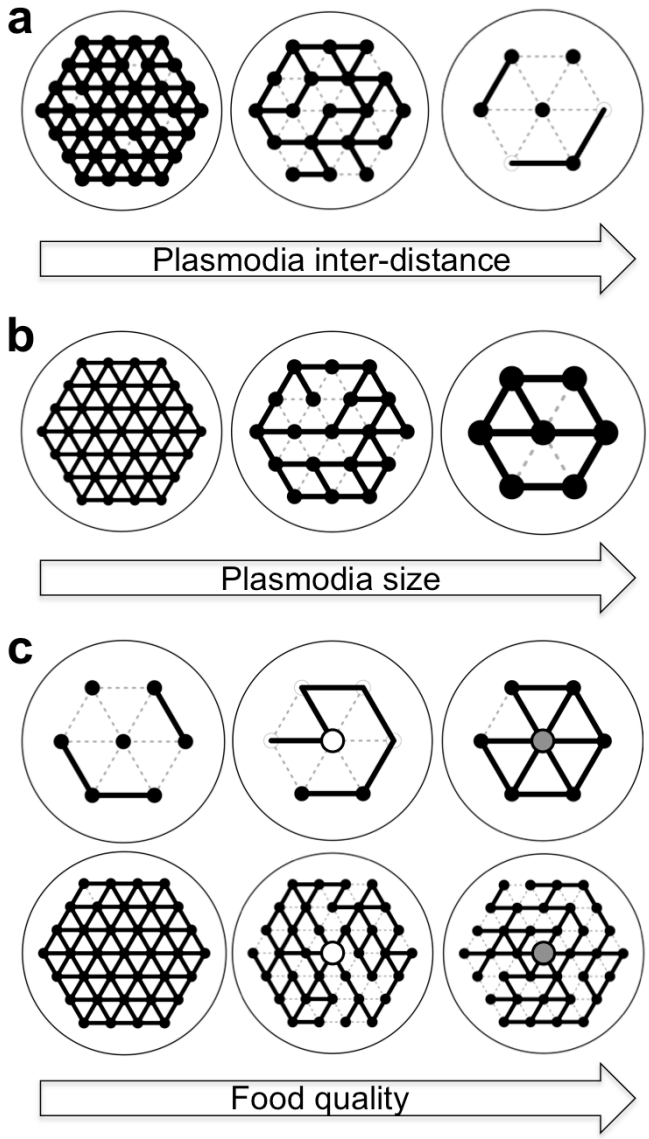

Figure 4: Schematic representations of the networks built by plasmodia in various contexts. (a) Role of inter-distance between plasmodia on the network. We tested three distances 1.5r, 3r and 6r. All plasmodia were of the same size 100mm^2. (b) Role plasmodia size on the network. We tested three plasmodia size 50, 100 and 270 mm^2. The distance between the plasmodia was 3r. (c) Role of the presence and quality of the food source on the network. We tested three situations: no food, low quality and high quality food. Two examples of configurations are illustrated: longest distance 6r (size 100mm^2) and smallest plasmodia 50mm^2 (distance 3r). Each schematic representation corresponds to one replicate. The black dots correspond to the plasmodia and the white ones represent the plasmodia that left their original location. The black lines correspond to the connections established between plasmodia and the grey dashed-lines represent the potential connections between plasmodia. The grey dots with a black heptagon correspond to the food source covered by plasmodia.

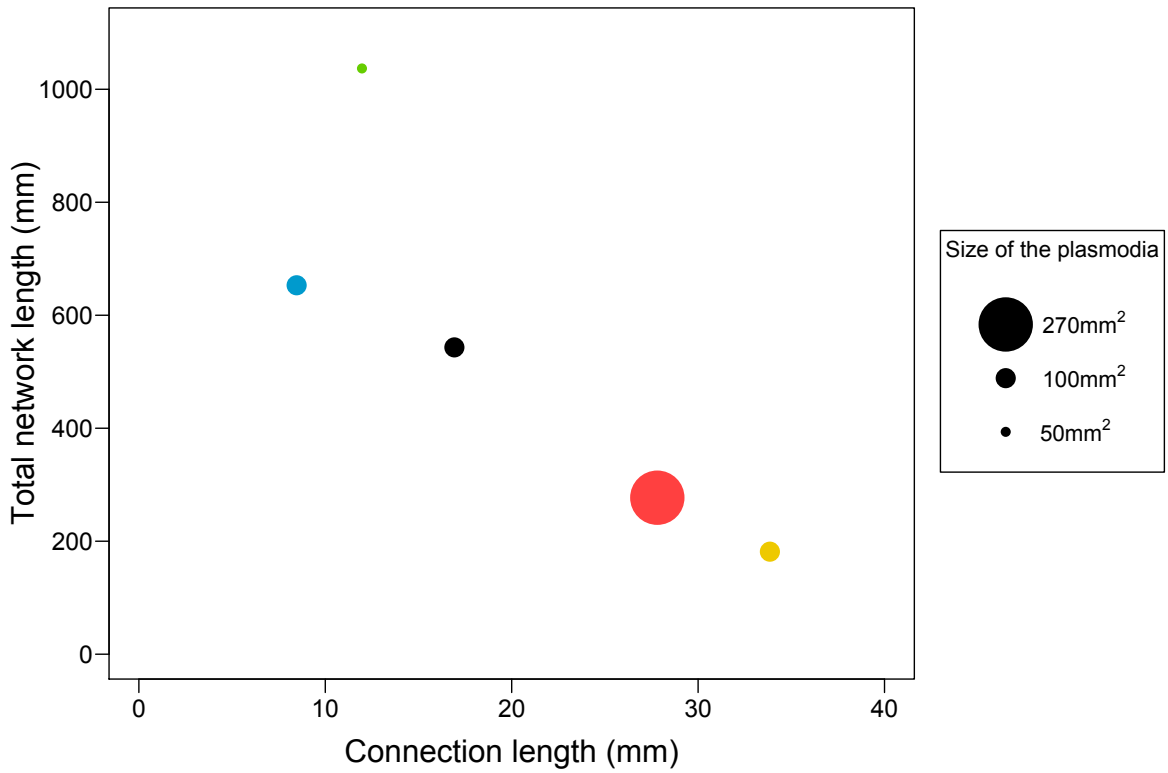

Figure 5: Total length of the network as function of the metric distance between plasmodia for the 5 configurations under exploration context. The formula for the total length of the network is shown in Table 1 Eq. 3. Each black dot corresponds to one configuration. The size of the dots are correlated to the size of the plasmodia in the corresponding configuration.

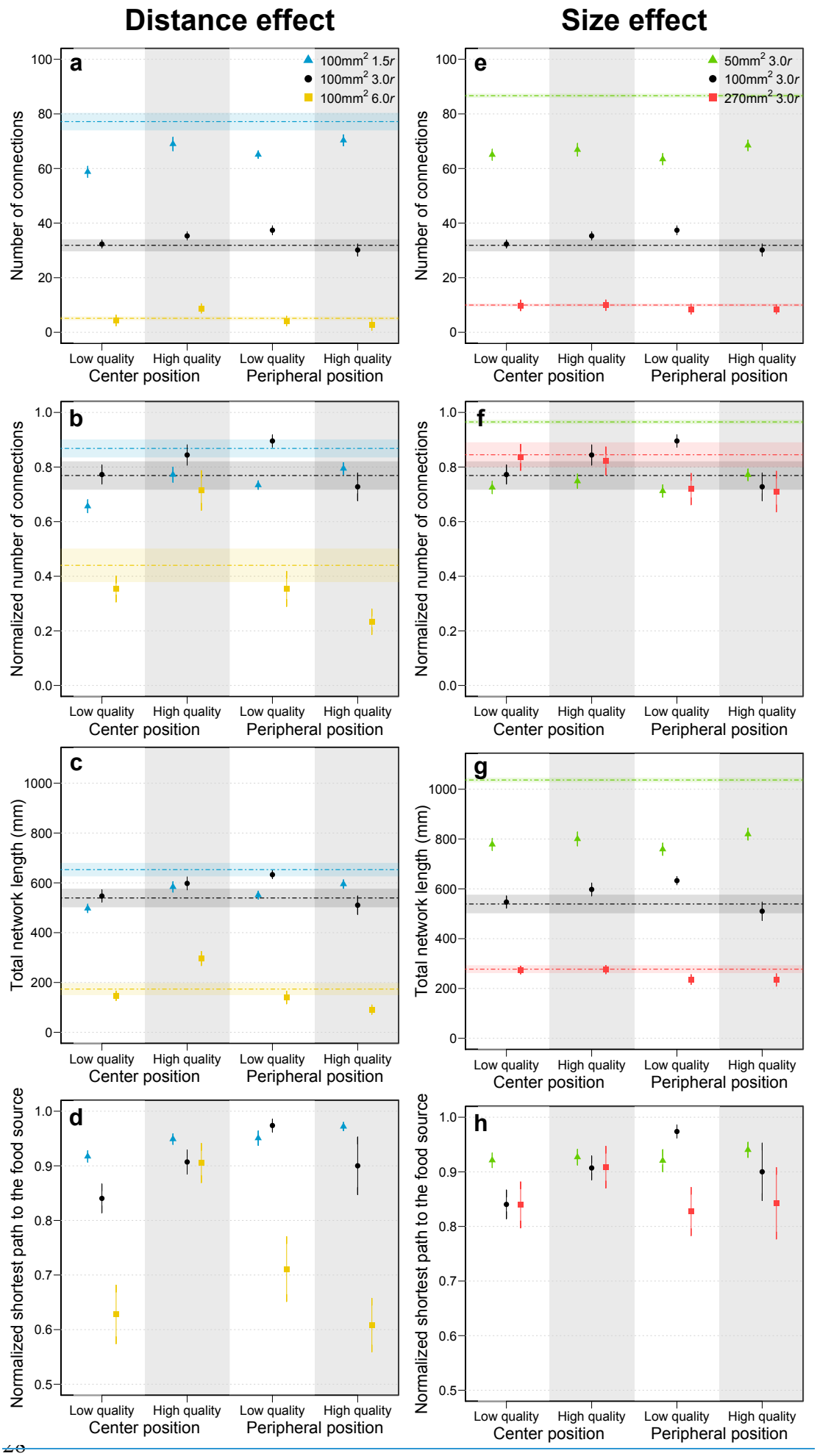
Distance effect
Size effect
a
b
c
d
e
f
g
h
100mm² 1.5r
100mm² 3.0r
100mm² 6.0r
50mm² 3.0r
100mm² 3.0r
270mm² 3.0r
Number of connections
Normalized number of connections
Total network length (mm)
Normalized shortest path to the food source
Low quality
High quality
Center position
Peripheral position

Figure 6: Connectivity parameters for the 5 configurations under foraging context. Number of connections (a and d) (Table 1 Eq. 1), normalized number of connections and (c and e) (Table 1 Eq. 2) total length of the network (c and f) (Table 1 Eq. 3) and normalized length of the network when (Table 1 Eq. 4) we varied the distance between plasmodia (a-d) or the size of the plasmodia (e-h). The dotted lines indicate the value obtained after 24h under exploration context. The food was either of low quality or high quality, and either at the centre or at the periphery. Error bars indicate ±95% CI.

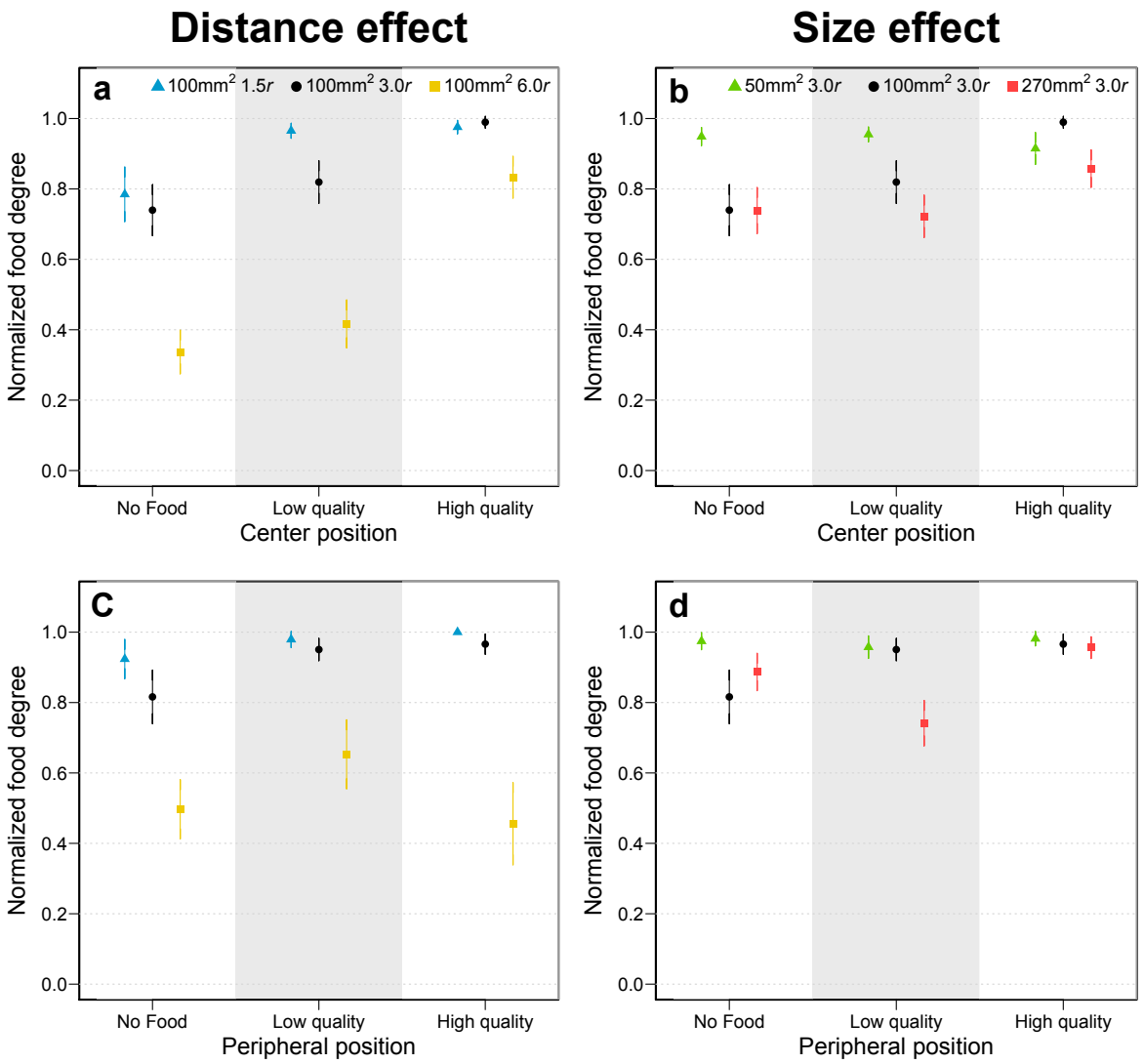

Figure 7: Normalized food degree. Normalized number of connections with the food (Table 1 Eq. 6) when we varied the distance between plasmodia (a, c) or the size of the plasmodia (b, d) and we moved the food from the centre (a-b) to the periphery (c-d). The food was either of low quality or high quality. Error bars indicate ±95% CI.

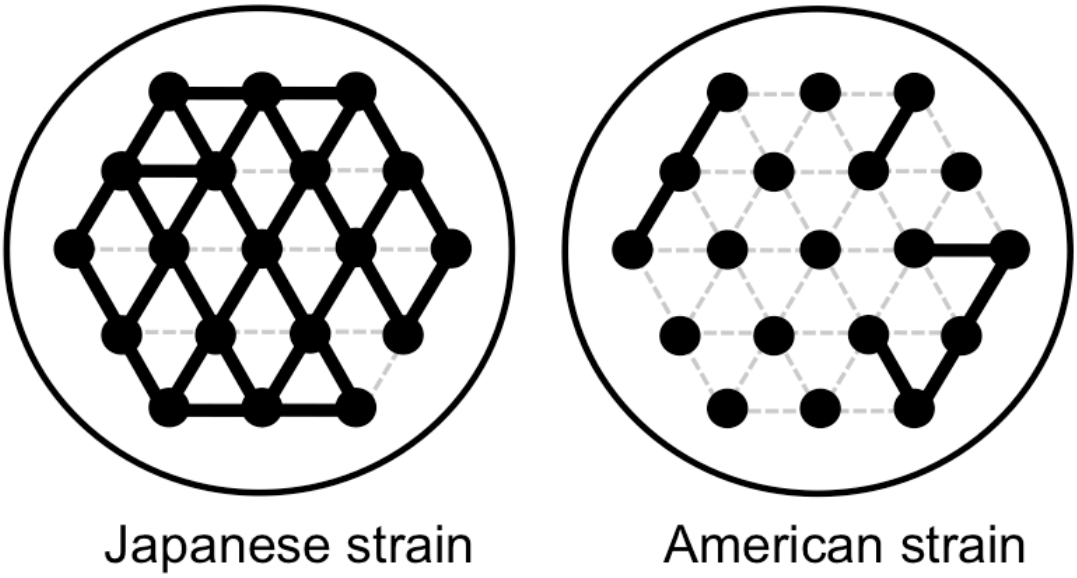

Figure 8: Schematic representations of the networks built by plasmodia of different strain. Two examples are illustrated with a distance between plasmodia equal to 3*r* and plasmodia size of 100mm^2. Each schematic representation corresponds to one replicate. The black dots correspond to the plasmodia. The black lines correspond to the connections established between plasmodia and the grey dashed-lines represent the potential connections between plasmodia.

	Measures	Formula	Equation
Network level	Number of connections	$NC = C_R$	(1)
	Normalized number of connections	$N_{NC} = \frac{NC}{T_{NC}}$	(2)
	Total network length	$NL = C_R \times l$	(3)
	Normalized total network length	$N_{NL} = \frac{NL}{T_{NC} \times l}$	(4)
Individual level	Plasmodium (or food) degree	$PD = C_P$	(5)
	Normalized plasmodium (or food) degree	$N_{PD} = \frac{PD}{T_{PD}}$	(6)
	Shortest path to the food source	SP	(7)
	Normalized shortest path to the food source	$N_{SP} = \frac{T_{SP}}{SP}$	(8)
Growth dynamics of individual plasmodium	Latency to contact a neighbouring plasmodium	LN	(9)
	Probability of contacting a neighbouring plasmodium during the isometric growth	$P_N = \frac{N_{P_{IG}}}{N}$	(10)

Table 1. Details of the measured parameters. C_R is the total number of direct connections established across all plasmodia in one replicate. T_{NC} corresponds to the number of connection for a triangulated network. l is the inter-distance between plasmodia. C_P is the number of connections observed between a plasmodium (or a food source) and its neighbouring plasmodia. T_{PD} corresponds to the number of connections between a plasmodium (or a food source) and its neighbouring plasmodia for a triangulated network. SP is the length of the shortest sequence of connections leading from one plasmodium to the food source. T_{SP} corresponds to the theoretical shortest path in the triangulated network. LN is the time it takes for one randomly chosen peripheral plasmodium to establish a connection with one of its direct neighbours. $N_{P_{IG}}$ is the number of randomly chosen plasmodia that contacted a neighbouring plasmodium while being in isometric growth phase. N corresponds to the number of plasmodia observed.

	Distance between plasmodia		
	1.5*r*	3*r*	6*r*
Latency to contact a neighbour (LN)	182.2±22.2	424.3±18.9	922.1±23.7
Normalised plasmodium degree (N_{PD})	0.87±0.03	0.77±0.02	0.44±0.03

	Size of the plasmodia		
	50mm^2	100mm^2	270mm^2
Latency to contact a neighbour (LN)	187.5±5.7	424.3±18.1	469.3±20.6
Normalised plasmodia degree (N_{PD})	0.96±0.01	0.77±0.02	0.84±0.02

720 **Table 2.** Mean latency to contact a neighbour (table 1 Eq. 9) and mean normalised
721 plasmodium degree (table 1 Eq. 6) under exploration context. Mean values are given with the
722 standard deviation (SD).
723

		Food source		
		High quality	Low quality	No food source
Configu)ration	6*r*	0.83	0.41	0.34
	1.5*r*	0.98	0.96	0.78
	3*r*/100mm^2	0.99	0.81	0.74
	50 mm^2	0.95	0.92	0.95
	270mm^2	0.85	0.72	0.73

724 **Table 3. Mean normalized food degree for the different configurations and quality of**
725 **food source**. The formula of the mean normalized food degree is shown in Table 1 Eq. 6
726